Journal of Medical Virology 56:151–158 (1998)

Polymorphisms of Thymidine Kinase Gene in Herpes Simplex Virus Type 1: Analysis of Clinical Isolates From Herpetic Keratitis Patients and Laboratory Strains

E. Kudo,[1] H. Shiota,[1]* T. Naito,[1] K. Satake,[1] and M. Itakura[2]

[1]Department of Ophthalmology, School of Medicine, University of Tokushima, Tokushima, Japan
[2]Otsuka Department of Clinical and Molecular Nutrition, School of Medicine, University of Tokushima, Tokushima, Japan

Drug-resistance of herpes simplex virus (HSV) is caused most frequently by mutation of the viral thymidine kinase (TK) gene. To elucidate the significance of detecting nucleotide changes of the TK gene for screening drug-resistant viruses, the frequency and variation of the genetic polymorphisms in the whole coding region of the TK gene were studied in 14 acyclovir-susceptible HSV type 1 (HSV-1) clinical isolates from 14 patients with epithelial herpetic keratitis. Two reference HSV-1 laboratory strains, McKrae and PH, and two acyclovir-resistant variants of the PH strain were also studied as controls. Polymerase chain reaction-single-strand conformation polymorphism (PCR-SSCP) and direct sequencing detected nucleotide differences at 24 positions, and amino acid substitutions at 12 codons in the TK gene of the examined viruses. Nucleotide diversity of 0.0029 per base (the average number of nucleotide substitutions of 3.3 per 1,131 base pairs) in the TK gene in the clinical isolates was comparable to 0.0037 per base of the whole HSV-1 genome in Japanese isolates reported previously. PCR-SSCP analysis of the acyclovir-resistant strains easily detected aberrantly shifted bands by comparing them with those of the parental strain, followed by the quick determination of mutated sequences. These results suggest that detection of nucleotide changes of the TK gene is useful for serial observation of persistent or recurrent HSV infection as observed in immunocompromised hosts, but that it is not useful for screening drug-resistant viruses from nonepidemic clinical isolates because of the comparable genetic polymorphisms in the TK gene as in the whole HSV-1 genome. ***J. Med. Virol. 56:151–158, 1998.***

KEY WORDS: HSV-1 isolates; PCR-SSCP; direct sequencing; genetic polymorphism; acyclovir resistance

INTRODUCTION

HSV infection of the eye is caused mostly by HSV type 1 (HSV-1) and it is a common cause of corneal blindness in both developed and developing countries [Reichman, 1984; Corey and Spear, 1986; Wilhelmus, 1987]. Nucleotide analogues, such as acyclovir (ACV) and ganciclovir (DHPG), are successfully and widely used as antiviral drugs with indications against many herpesvirus diseases, including herpetic ocular diseases [Richards et al., 1983; Furman, 1987; Shiota et al. 1987; Keating, 1992]. Because these drugs are activated intracellularly through phosphorylation by thymidine kinase (TK) of HSV, both experimentally established and clinically isolated drug-resistant HSV are caused most frequently by mutations in the viral TK gene [Englund et al., 1990; Coen, 1991; Nugier et al., 1992; Collins, 1993; Fife et al., 1994].

Plaque reduction assay, quantitative dye-uptake assay, and their modifications are used currently to detect drug-resistant viruses [McLaren et al., 1983; Langliois et al., 1986; Englund et al., 1990; Nugier et al., 1992; Fife et al., 1994]. Because the viruses in question are inoculated by these methods into suitable cells such as Vero cells or human embryonic lung fibroblasts, cell culture must be available for virus inoculation. The direct mutation detection of the TK gene, e.g., screening by PCR-SSCP analysis followed by sequencing, is an alternative approach because it does not require cell

*Correspondence to: Dr. Hiroshi Shiota, Department of Ophthalmology, School of Medicine, University of Tokushima, 3-18-15, Kuramoto-cho, Tokushima 770, Japan. E-mail: shiota@clin.med.tokushima-u.ac.jp

Accepted 11 March 1998

culture and, besides, it may be suitable for automation. The ability of SSCP analysis to detect a variety of mutations in the TK gene, even in a mixture of viruses, is also advantageous.

Sakaoka et al. [1994] studied genomic polymorphisms in HSV-1 isolates from six countries using the presence or absence of 225 restriction endonuclease sites scattering throughout the HSV-1 genome, and estimated the value of nucleotide diversity (average nucleotide differences per nucleotide) of the whole HSV-1 genome to be 0.0037 for isolates from Japan, 0.0022 from Korea, 0.0029 from China, 0.0044 from Sweden, 0.0046 from the United States, and 0.0056 from Kenya. The average number of nucleotide differences in the coding region of the TK gene (1,131 bp) in Japanese HSV-1 isolates is thus expected to be 4.2 (= 1,131 × 0.0037) in the case that the rate of genetic polymorphism of the TK gene is equivalent to that of the whole HSV-1 genome. But the actual number of nucleotide differences in the TK gene may be smaller, because herpetic diseases are usually transmitted through inoculation of virions in peripheral tissue and TK activity is necessary for reactivation of the latent HSV in sensory ganglion neurons [Efstathiou et al., 1989]. Within the supposed limited number of polymorphisms of the TK gene in HSV clinical isolates, a combination of SSCP analysis and direct sequencing of the TK gene is expected to be useful for detecting drug-resistant viruses. A list of polymorphisms of the TK gene in clinical isolates should also help identifying drug-resistant TK mutations. Here we report the frequency and variety of polymorphisms of the TK gene in 14 ACV-susceptible HSV-1 strains isolated from the eyes of patients with active herpetic keratitis and 2 HSV-1 laboratory strains, PH and McKrae. Two ACV-resistant strains derived from the PH strain were also studied as examples of resistant viruses.

METHODS AND MATERIALS

Cells and Viruses

Vero cells were cultured in Eagle's minimum essential medium (MEM) (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 10% fetal calf serum (FCS) (Flow Laboratories Inc., Irvine, VA), 0.001 M L-glutamine, 60 μg/ml kanamycin, and 0.2% $NaHCO_3$. Vero cells were used for isolation, titration, and plaque inhibition assay of viruses.

Fourteen HSV-1 isolates were obtained from the eyes of 14 patients with herpetic keratitis. These patients were diagnosed with epithelial herpetic keratitis at Tokushima University Hospital and were epidemiologically unrelated. None had received any antiviral therapy prior to the isolation of viruses. Corneal scrapings of the patients were inoculated onto Vero cell monolayers. Aliquots of culture supernatants containing viral particles were stored at −80°C until use for plaque assay and extraction of DNA. To avoid effects of cell culture passage on viral populations, viral isolates of less than three passages were used. All viruses isolated were collected between August 1988 and January 1993, and confirmed as HSV-1 by a direct immunofluorescent method using type-specific monoclonal antibodies (MicroTrack, Syva Co., San Jose, CA). Two HSV-1 laboratory strains, McKrae and PH, were also examined in the same way for comparison. Two ACV-resistant variants of the PH strain established previously in vitro were also used as examples of resistant viruses [Shiota et al., 1996]. Briefly, the PH strain was grown and passed several times in Vero cells in medium containing 10^{-6} M ACV or 10^{-7} M DHPG. The descendent viruses were cloned, and the resistant strains, named PH-ACV^r and PH-DHPG^r, were established from these viral clones. The PH-DHPG^r strain was also resistant to ACV.

Plaque Inhibition Assay

ACV was a gift from Glaxo Wellcome p1c, U.K. Vero cell monolayers in 60 mm culture dishes were inoculated with 200 plaque-forming units (PFU) of the virus under test and then overlaid with 4 ml of Eagle's MEM containing 1% FCS, 0.5% methylcellulose, and an appropriate amount of ACV at serial half-log_{10} dilutions. After incubation for 3 days in an atmosphere of 5% CO_2 in air, the cultures were stained with a solution of 1% crystal violet in 10% formalin. The concentration of drug-inhibiting plaque formation by 50% (ID_{50}) was determined from an analysis of the number of plaques in duplicate plates at each dilution of ACV compared with the number of plaques obtained with duplicate plates without ACV.

DNA Samples

Twenty microliters of culture supernatant containing about 2×10^6 PFU of each virus were mixed with 180 μl of proteinase K solution (250 μg/ml proteinase K, 0.25% SDS, 5 mM EDTA, and 10 mM Tris-HCl, pH 8.0), and incubated at 56°C for 2 hr. After inactivating proteinase K by incubation at 95°C for 10 min, the sample mixture was diluted 10 times with ultrapure water and used as a DNA sample for PCR analysis.

PCR-SSCP Analysis

PCR-SSCP analysis was carried out by the method of Orita et al. [1989] with a slight modification. Seven pairs of primers were designed based on sequences reported previously [McKnight, 1980; Kit et al., 1983; McGeoch et al., 1988] (Table I and Fig. 1). Each primer pair was designed to amplify a part of the TK gene of HSV-1 but not to amplify that of HSV type 2 (HSV-2). Ten microliters of the PCR mixture contained 1 μl of DNA sample, 10 pmol each of primers, 0.25 nmol each of dNTPs, 0.1 μl of [α-^{32}P]dCTP (110 TBq/mmol; 370 MBq/ml, Amersham, Buckinghamshire, U.K.) and 0.25 units of Taq DNA polymerase (Takara, Ohtsu, Japan) in the buffer supplied by Takara. After denaturing template DNA by heating at 95°C for 1 min, 40 cycles of the reaction at 95°, 55°, and 72°C for 30 sec, each was carried out in a thermal cycler (GeneAmp PCR System 2400, Perkin-Elmer, Norwalk, CT). The resultant PCR product in 10 μl was then mixed with 8 μl of a form-

TABLE I. Primers Used for PCR-SSCP Analysis

Part	Primer[a]	Sequence	Position[b]
1	Pu1	5′-TTCGGCCAGCGCCTTGTAG-3′	−27 → −9
	Pd1	5′-GTGGGCATTTTCTGCTCCAG-3′	143 → 124
2	Pu2	5′-CAACAAAAAGCCACGGAAGTC-3′	103 → 120
	Pd2	5′-GATATCTCACCCTGGTCGAG-3′	338 → 319
3	Pu3	5′-GGGGGCTTCCGAGACAATC-3′	273 → 291
	Pd3	5′-GCATGTGAGCTCCCAGCCT-3′	455 → 437
4	Pu4	5′-CCGTGACCGACGCCGTTCT-3′	397 → 415
	Pd4	5′-CAGGCGGTCGATGTGTCTGT-3′	651 → 632
5	Pu5	5′-GCCCGGCACAAACATCGTG-3′	594 → 602
	Pd5	5′-AAATAACGTGTCCCCGATATGG-3′	867 → 846
6	Pu6	5′-AGGATTGGGGACAGCTTTCG-3′	770 → 789
	Pd6	5′-GGAGGCGTTTGGCCAAGAC-3′	958 → 940
7	Pu7	5′-ACGGCGACCTGTATAACGTG-3′	902 → 921
	Pd7	5′-GTTCCTTCCGGTATTGTCTCC-3′	1161 → 1141

[a]Pu and Pd = an upstream primer and a downstream primer, respectively.
[b]According to the sequence reported by McGeoch et al. [1988].

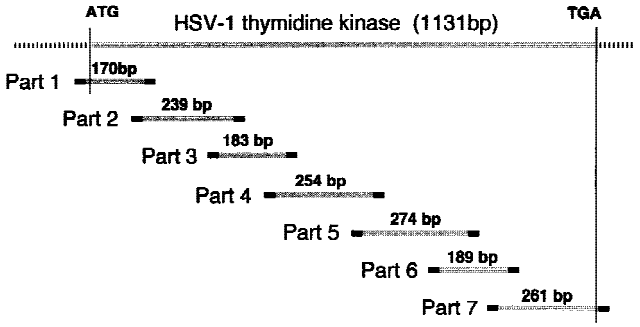

Fig. 1. Schematic presentation of the location of DNAs amplified by PCR. Bold lines on both sides of each DNA indicate the pairs of primers. Sizes of amplified DNAs are shown in base pairs.

amide dye mixture (95% formamide, 0.05% xylene cyanol, and 0.05% bromophenol blue) and 2 μl of 10 mM EDTA in 0.5 N NaOH, heated at 95°C for 3 min, and applied (1 μl/lane) to 6% polyacrylamide gel containing 45 mM Tris-borate (pH 8.3) and 1 mM EDTA. All the PCR products were analyzed by PCR-SSCP at three glycerol concentrations of 0, 5, or 10%. Electrophoresis was undertaken at 20 W for 3 to 6 hr with cooling by a fan at room temperature. The gel was dried on filter paper and exposed to an X-ray film with an intensifying screen overnight at room temperature.

DNA Sequencing

For direct sequencing of the TK gene, seven pairs of primers, including one with the M13 forward consensus sequence (5′-TGTAAAACGACGGCCAGT-3′) attached to the 5′ end of the upstream PCR primers and another with the M13 reverse consensus sequence (5′-CAGGAAACAGCTATGACC-3′) attached to the 3′ end of the downstream PCR primers, were used. The portion of the dried polyacrylamide gel containing DNA with altered mobility found on SSCP analysis was cut out, and DNA was extracted overnight from the gel with 50 μl of water at 37°C. PCRs were carried out under the same conditions as for PCR-SSCP analysis as described above except for omitting [α-^{32}P]dCTP and using the extracted DNAs from the dried gel as templates. Nucleotide sequences were determined by the fluorescence-based dideoxy sequencing method with a dye primer cycle sequencing FS ready reaction kit (Perkin-Elmer) and an automated DNA sequencer model 377 (Perkin-Elmer). Both the sense and antisense sequences were determined in every case.

RESULTS

Sensitivity of Clinical Isolates and Laboratory Strains to ACV

ID_{50} values of 14 clinical HSV-1 isolates, two laboratory strains, and two derivative resistant strains were measured by a plaque inhibition assay (Fig. 2 and Table II). All clinical isolates and laboratory strains showed ID_{50} values of less than 6.66 μM (= 1.5 μg/ml), indicating that they were susceptible to ACV [Nugier et al., 1992; Fife et al., 1994]. In contrast, two derivative resistant strains, PH-ACV^r and PH-DHPG^r, showed ID_{50} values of more than 30 μM (= 6.76 μg/ml).

Polymorphisms of the TK Gene in Clinical Isolates and Laboratory Strains

The entire coding regions of the TK gene of 14 HSV-1 clinical isolates and two laboratory strains, McKrae and PH, were screened for genetic polymorphisms by PCR-SSCP analysis in three gel conditions. In every part of the TK gene, the amplified DNAs were classified according to their migration patterns (Fig. 3). Three migration patterns were observed in parts 1 through 6, and four patterns in part 7. The migration patterns on PCR-SSCP analysis were reproducible in separate experiments. One representative DNA was selected from the amplified DNAs showing the identical migration pattern, and its nucleotide sequence was determined by direct sequencing and compared with that of the HSV-1 strain 17 [McGeoch et al., 1988] (Table III). Heterogeneous polymorphisms were not observed in any of clinical isolates. Consequently, in the clinical isolates, nucleotide substitutions were found at eight positions, and amino acid substitutions at four codons. Two laboratory strains had nucleotide substi-

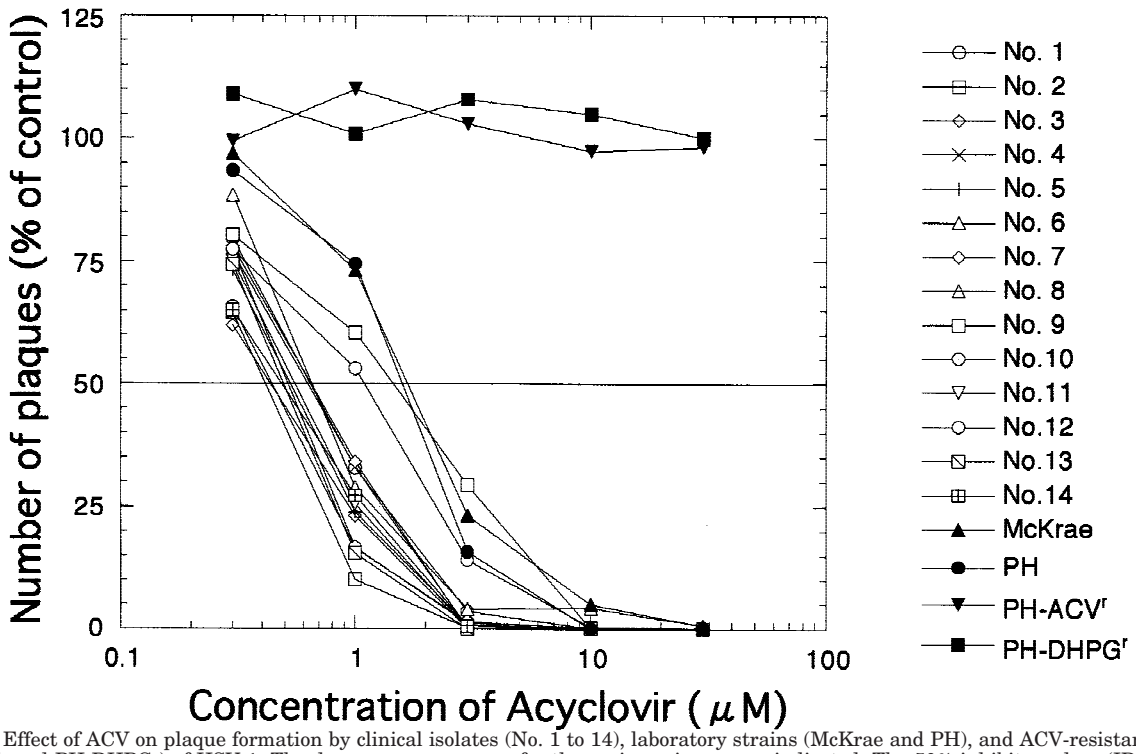

Fig. 2. Effect of ACV on plaque formation by clinical isolates (No. 1 to 14), laboratory strains (McKrae and PH), and ACV-resistant strains (PH-ACV^r and PH-DHPG^r) of HSV-1. The dose–response curves for the various viruses are indicated. The 50% inhibitory dose (ID_{50}) for all clinical isolates and laboratory strains are less than 1.5 μg/ml (= 6.66 μM), indicating that they are susceptible to ACV.

TABLE II. Migration Patterns on PCR-SSCP Analysis of the TK Gene and ID_{50} Values of HSV-1 Clinical Isolates and Laboratory Strains

Group	Virus	Pattern[a]	ID_{50} (μM)[b]
I	No. 1	−−−−−−−	0.65
	No. 2	−−−−−−−	0.40
	No. 4	−−−−−−−	0.62
II	No. 9	−−b−−−−	1.40
	No. 12	−−b−−−−	1.10
	No. 14	−−b−−−−	0.48
III	No. 3	bb−−−−−	0.42
	No. 5	bb−−−−−	0.52
	No. 6	bb−−−−−	0.65
	No. 7	bb−−−−−	0.65
	No. 8	bb−−−−−	0.51
	No. 13	bb−−−−−	0.48
IV	No. 11	bb−b−−−	0.55
V	No. 10	bb−−−−b	0.44
	McKrae	cc−−bbc	1.60
	PH	−bcccd	1.60

[a]Migration patterns of the amplified DNAs on PCR-SSCP analysis from part 1 (left end) through part 7 (right end) are summarized. "–" indicates "a" pattern.
[b]ID_{50} values of acyclovir.

tutions at 16 other positions and amino acid substitutions at eight other codons. In addition, strain 17 had nucleotide substitutions at five positions and an amino acid substitution at one codon, both of which were not found in the TK gene in the clinical isolates and two laboratory strains. The clinical isolates were classified into five groups with regard to the combination of polymorphism patterns on SSCP analyses (Table II). The sequence data of a virus or a viral group in every combination were compared with each other, and the differences in nucleotides and amino acids were summarized (Table IV). The average number of nucleotide substitutions was 3.3 for the clinical isolates, and 5.3 for all 16 examined viruses per 1,131 bp of the TK gene. The value of nucleotide diversity was thus 0.0029 (= 3.3/1,131) for the clinical isolates, and 0.0047 (= 5.3/1,131) for all 16 viruses.

Causative Mutation of Two ACV-Resistant Strains

PCR-SSCP analysis of the TK gene of PH-ACV^r and PH-DHPG^r showed altered migration patterns in polyacrylamide gel in part 4 and part 2, respectively (Fig. 4). Sequencing revealed a nonsense mutation on codon 176 [Arg(CGA)→stop(TGA)] of the TK gene of PH-ACV^r. Strain PH-DHPG^r was formerly reported to have a nonsense mutation at codon 95 [Glu(GAG)→stop(TAG)] of the TK gene [Shiota et al., 1996].

DISCUSSION

In this study, we found nucleotide substitutions at 24 positions, and amino acid substitutions at 12 codons of the TK gene in 14 HSV-1 clinical isolates and two laboratory strains, McKrae and PH. Since all these viruses were susceptible to ACV, all nucleotide substitutions found should represent genetic polymorphisms that do not overtly affect the TK activity. The chance of overlooking the polymorphisms should be low, because the sensitivity of PCR-SSCP analysis for detecting differences of nucleotide sequence in DNA fragments of 100-

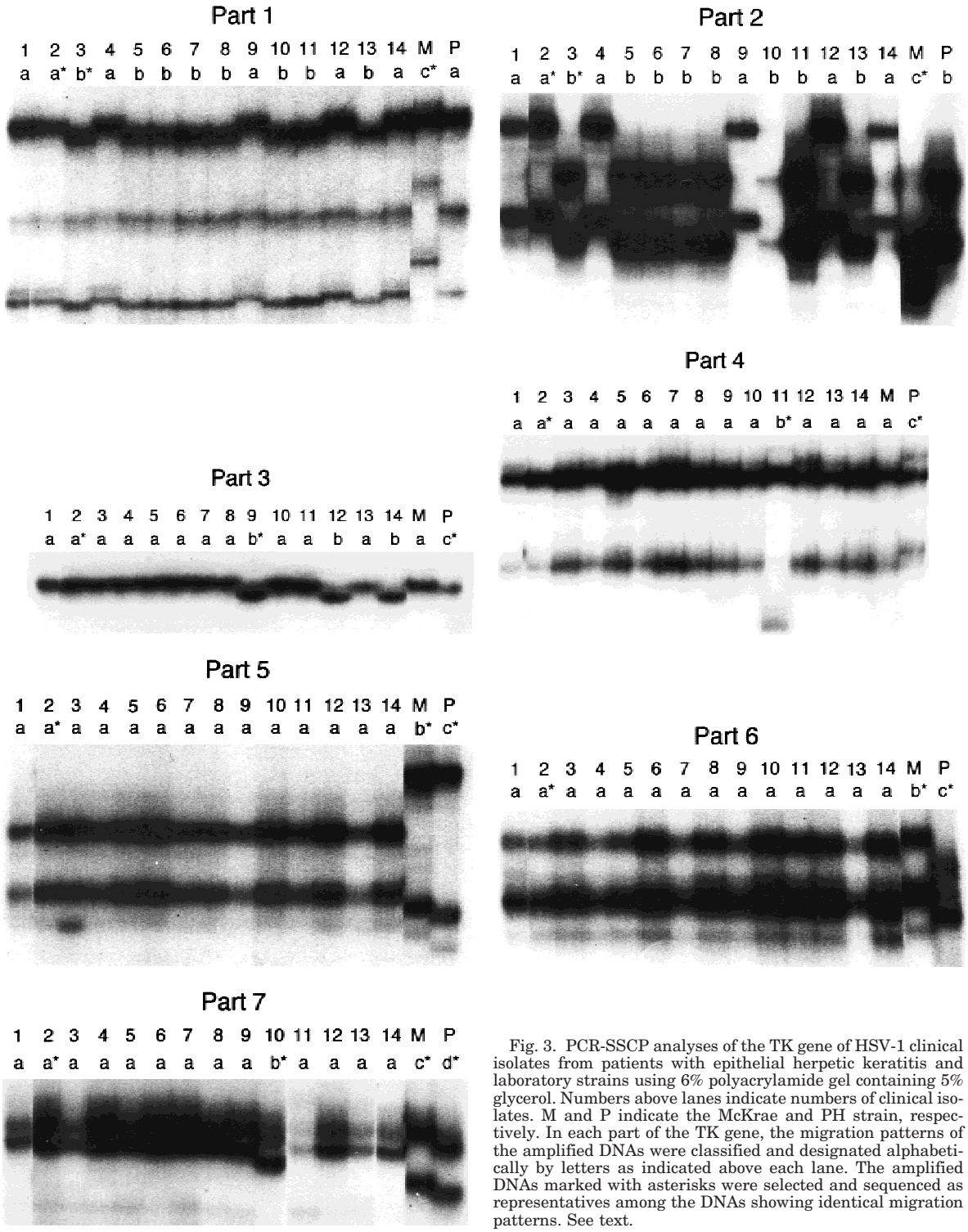

Fig. 3. PCR-SSCP analyses of the TK gene of HSV-1 clinical isolates from patients with epithelial herpetic keratitis and laboratory strains using 6% polyacrylamide gel containing 5% glycerol. Numbers above lanes indicate numbers of clinical isolates. M and P indicate the McKrae and PH strain, respectively. In each part of the TK gene, the migration patterns of the amplified DNAs were classified and designated alphabetically by letters as indicated above each lane. The amplified DNAs marked with asterisks were selected and sequenced as representatives among the DNAs showing identical migration patterns. See text.

to 300-bp, under the three different gel conditions used in the present study, is very high [Orita et al., 1989].

In the clinical isolates, the average number of nucleotide differences in the TK gene was 3.3 per 1,131 bp, which was comparable to the expected value of 4.2. This finding suggests that the rate of nucleotide differences in the TK gene is indeed comparable to that of the whole HSV-1 genome. Moreover, the laboratory strains PH, McKrae, and strain 17 had diverse genetic polymorphisms that were not found in our clinical isolates. The PCR-SSCP screening of the TK gene is not expected to be useful for detecting ACV-resistant virus because frequent polymorphisms interfere with the detection of drug-resistant mutations. Migration bands

TABLE III. Relations Between Migration Patterns on PCR-SSCP Analyses and Nucleotide Sequences of the TK Gene

Part 1

	Codon[a]				
Pattern	6	8	23	34	36
a	TGC (Cys)	CAA (Gln)	AGC (Ser)	CAG (Gln)	GAA (Glu)
b	G–– (Gly)	––G	–––	–––	–––
c	–––	–––	–A– (Asn)	–––	A–– (Lys)
Strain 17[b]	–––	–––	–A– (Asn)	––A	A–– (Lys)

Part 2

	Codon[a]			
Pattern	42	57	89	91
a	CTG (Leu)	CCT (Pro)	CAG (Gln)	CTG (Leu)
b	–C– (Pro)	––C	–G– (Arg)	–––
c	–––	––C	–G– (Arg)	T––
Strain 17[b]	–––	––C	–G– (Arg	T––

Part 3

	Codon[a]	
Pattern	116	138
a	GAC (Asp)	GTT (Val)
b	––T	–––
c	–––	––G
Strain 17[b]	–––	–––

Part 4

	Codon[a]		
Pattern	171	195	199
a	TGC (Cys)	CCG (Pro)	CCC (Pro)
b	–––	––T	–––
c	––T	–––	––G
Strain 17[b]	–––	–––	–––

Part 5

	Codon[a]						
Pattern	239	240	265	267	268	280	281
a	TAC (Tyr)	GGG (Gly)	ACG (Thr)	GTG (Val)	CCG (Pro)	CCA (Pro)	CGA (Arg)
b	–––	–A– (Glu)	–––	–––	–––	–––	–A– (Gln)
c	–––	–A– (Glu)	–––	T–– (Leu)	A–– (Thr)	––G	–––
Strain 17[b]	––T	–––	G–– (Ala)	–––	–––	–––	–––

Part 6

	Codon[a]						
Pattern	267	268	280	281	286	305	311
a	GTG (Val)	CCG (Pro)	CCA (Pro)	CGA (Arg)	GAC (Asp)	TAT (Tyr)	GCC (Ala)
b	–––	–––	–––	–A– (Gln)	–––	–––	–––
c	T–– (Leu)	A–– (Thr)	––G	–––	––A (Glu)	––C	–––
Strain 17[b]	–––	–––	–––	–––	–––	–––	––T

Part 7

	Codon[a]		
Pattern	323	355	376
a	CAC (His)	CCA (Pro)	AAC (Asn)
b	–––	–A– (Gln)	–––
c	–––	––C	–––
d	–––	–––	CC– (Pro)
Strain 17[b]	––T	–––	–––

[a]According to the sequence reported by McGeoch et al. [1988]. Only codons of the TK gene differing among the examined viruses or from those of the HSV-1 strain 17 are displayed. Only nucleotides and amino acids differing from those in pattern "a" are displayed by letters.
[b]HSV-1 strain 17 [McGeoch et al., 1988].

with abnormal mobilities were easily detected by PCR-SSCP analyses of the TK gene of PH-ACV^r and PH-DHPG^r on an autoradiogram, and the mutated sequences were determined quickly. Therefore, the present method should be useful when control parental strains are available for comparison, for example, during serial observation of persistent or recurrent HSV infection as seen in immunocompromised hosts, such as patients with AIDS and recipients with organ transplantation. Drug-resistant HSV strains are often re-

TABLE IV. Numbers of Substituted Nucleotides and Amino Acids Between Viruses or Virus Groups Studied

Compared with	Number of substituted nucleotides (amino acids)						
	Clinical isolates[a]					Strains	
	Group I	Group II	Group III	Group IV	Group V	McKrae	PH
Group II	1 (0)						
Group III	5 (3)	6 (3)					
Group IV	6 (3)	7 (3)	1 (0)				
Group V	6 (4)	7 (4)	1 (1)	2 (1)			
McKrae	8 (5)	9 (5)	9 (6)	10 (6)	10 (7)		
PH	14 (7)	15 (7)	13 (6)	14 (6)	14 (7)	16 (8)	
Strain 17[b]	10 (4)	11 (4)	11 (5)	12 (5)	12 (6)	7 (3)	20 (9)

[a]Groups I to V are the same as those in Table II.
[b]HSV-1 strain 17 [McGeoch et al., 1988].

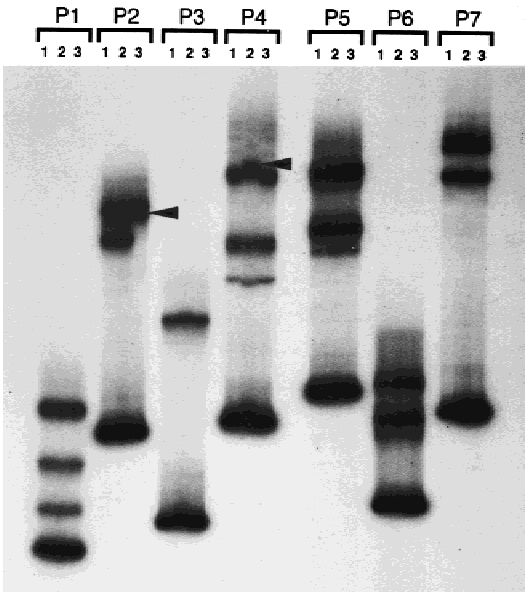

Fig. 4. PCR-SSCP analysis of the TK genes of the PH, PH-ACV^r, and PH-DHPG^r strains. The analyses were performed under the same conditions as those in Figure 3. Numbers above brackets indicate numbers of parts of the TK gene amplified using the primers listed in Table I. The PCR products amplified using DNA of the PH, PH-ACV^r, and PH-DHPG^r strains were denatured and electrophoresed in lanes 1, 2, and 3, respectively, under each bracket. In each lane, the lowest band represents double-stranded DNA, and the upper two bands represent separated single-stranded DNAs. Part 3 of the TK gene gave only one upper band because the two single-stranded DNAs have the same mobility in these conditions. Note that parts 2 and 4 of the TK gene of PH-DHPG^r and PH-ACV^r strains, respectively, showed altered migration patterns (arrow heads).

ported to be recovered from these patients receiving prolonged antiviral drug therapy [Englund et al., 1990; Coen, 1991; Nugier et al., 1992; Collins, 1993; Fife et al., 1994].

It is noteworthy that none of the amino acid substitutions found in this study was within the putative active centers of the TK enzyme, i.e., ATP binding site (residues 51 to 63), nucleoside binding site (168 to 176), and the residue 336 [Darby et al., 1986]. Balasubramaniam et al. [1990] compared sequences of TK genes of 12 viruses of herpesviridae and found six highly conserved sites, i.e., site 1 (residues 56 to 62), site 2 (83 to 88), site 3 (162 to 164), site 4 (171 to 173), site 5 (216 to 222), and site 6 (284 to 289). Only an amino acid substitution at residue 286 [Asp(GAC)→Glu(GAA)] of the TK of the PH strain was within the six conserved sites. This substitution should not affect the TK activity, because the same substitution was also found in an HSV-1 reference laboratory strain VR-3 [Suzutani et al., 1995]. PCR-SSCP analyses using primer sets specific for these important sites may be useful for screening ACV-resistant viruses of the TK-altered type mentioned below, although further study using a larger number of clinical isolates is needed to clarify this point.

In both laboratory and clinical investigations, the most frequently isolated ACV-resistant HSV strains are TK-defective mutants that produce a nonfunctional TK enzyme due to premature termination [Englund et al., 1990; Coen, 1991; Nugier et al., 1992; Collins, 1993; Fife et al., 1994]. Viral DNA polymerase is another enzyme involved in the mechanism of action of ACV. Mutations in the viral gene encoding DNA polymerase also cause resistance to ACV, but such mutations are observed rarely in clinical isolates [Parker et al., 1987; Sacks et al., 1989]. TK-altered mutants of HSV exhibit ACV resistance due to mutation-derived altered substrate binding properties of TK. In contrast with the TK-defective mutants, the TK-altered mutants retain their ability for reactivation from latent infections and their virulence approaches that of the parental strains [Darby et al., 1981; Nugier et al., 1991]. The TK-altered mutants were reported to cause severe prolonged diseases in immunocompromised hosts [Englund et al., 1990; Nugier et al., 1991, 1992]. Moreover, mixtures of TK-altered and wild-type viruses in mouse models caused serious herpetic disease that was difficult to treat [Ellis et al., 1989; Coen, 1991]. The TK-altered mutants are thus significant clinically, although they are fewer than the TK-deficient mutants. To clarify the causative mutations in the TK-altered mutants, it is necessary to differentiate pathogenic mutations from innocuous polymorphisms. It is thus very important to accumulate data on polymorphisms found in the TK gene of clinical isolates. The combination of PCR-SSCP and direct sequencing used in the present study will also be useful for further studies on this subject.

In conclusion, the nucleotide diversity of 0.0029 per

base (an average nucleotide polymorphism of 3.3 per 1,131 bp) of the TK gene in 14 clinical isolates of HSV-1 was comparable to 0.0037 per base of the whole HSV-1 genome. Detecting nucleotide changes of the TK gene is useful for serial observation of persistent or recurrent HSV infection as seen in immunocompromised hosts, but it is not useful for screening drug-resistant viruses from nonepidemic clinical isolates. The list of polymorphisms of the TK gene presented above should help identifying drug-resistant TK mutations.

ACKNOWLEDGMENTS

We thank Glaxo Wellcome p1c., U.K., for providing ACV. This study was supported in part by a grant from the Uehara Memorial Foundation to the Department of Ophthalmology and a grant from Otsuka Pharmaceutical Co., Ltd. to Otsuka Department of Clinical and Molecular Nutrition, School of Medicine, University of Tokushima.

REFERENCES

Balasubramaniam NK, Veerisetty V, Gentry GA (1990): Herpesviral deoxythymidine kinases contain a site analogous to the phosphoryl-binding arginine-rich region of porcine adenylate kinase; Comparison of secondary-structure predictions and conservation. Journal of General Virology 71:2979–2987.

Coen DM (1991): The implications of resistance to antiviral agents for herpesvirus drug targets and drug therapy. Antiviral Research 15:287–300.

Collins P (1993): Mechanisms of herpes virus resistance. Annals of Medicine 25:441–445.

Corey L, Spear PG (1986): Infections with herpes simplex viruses. New England Journal of Medicine 314:749–757.

Darby G, Field HJ, Salisbury SA (1981): Altered substrate specificity of herpes simplex virus thymidine kinase confers acyclovir-resistance. Nature 289:81–83.

Darby G, Larder B, Inglis MM (1986): Evidence that the "active centre" of the herpes simplex virus thymidine kinase involves an interaction between three distinct regions of the polypeptide. Journal of General Virology 67:753–758.

Efstathiou S, Kemp S, Darby G, Minson C (1989): The role of herpes simplex virus type 1 thymidine kinase in pathogenesis. Journal of General Virology 70:869–879.

Ellis MN, Waters R, Hill EL, Lobe DC, Selleseth DW, Barry DW (1989): Orofacial infection of athymic mice with defined mixtures of acyclovir-susceptible and acyclovir-resistant herpes simplex virus type 1. Antimicrobial Agents and Chemotherapy 33:304–310.

Englund JA, Zimmerman ME, Swierkosz EM, Goodman JL, Scholl DR, Balfour HH (1990): Herpes simplex virus resistant to acyclovir: A study in a tertiary care center. Annals of Internal Medicine 112:416–422.

Fife KH, Crumpacker CS, Mertz GJ, Hill EL, Boone GS et al. (1994): Recurrence and resistance patterns of herpes simplex virus following cessation of ≥6 years of chronic suppression with acyclovir. Journal of Infectious Diseases 169:1338–1341.

Furman PA (1987): Chemotherapy of herpesvirus infections. Current Eye Research 6:213–219.

Keating MR (1992): Antiviral agents. Mayo Clinic Proceedings 67: 160–178.

Kit S, Kit M, Qavi H, Trkula D, Otsuka H (1983): Nucleotide sequence of the herpes simplex virus type 2 (HSV-2) thymidine kinase gene and predicted amino acid sequence of thymidine kinase polypeptide and its comparison with the HSV-1 thymidine kinase gene. Biochemica et Biophysica Acta 741:158–170.

Langlois M, Allard JP, Nugier F, Aymard M (1986): A rapid and automated colorimetric assay for evaluating the sensitivity of herpes simplex strains to antiviral drugs. Journal of Biological Standardization 14:201–211.

McGeoch DJ, Dalrymple MA, Davidson AJ, Dolan A, Frame MC, McNab D, Perry LJ, Scott JE, Taylor P (1988): The complete DNA sequence of the long unique region in the genome of herpes simplex virus type 1. Journal of General Virology 69:1531–1574.

McKnight SL (1980): The nucleotide sequence and the transcript map of the herpes simplex thymidine kinase gene. Nucleic Acids Research 8:5949–5964.

McLaren C, Ellis MN, Hunter GA (1983): A colorimetric assay for the measurement of the sensitivity of herpes simplex viruses to antiviral agents. Antiviral Research 3:223–234.

Nugier F, Collins P, Larder BA, Langlois M, Aymard M, Darby G (1991): Herpes simplex virus isolates from an immunocompromised patient who failed to respond to acyclovir treatment express thymidine kinase with altered substrate specificity. Antiviral Chemistry and Chemotherapy 2:295–302.

Nugier F, Colin JN, Aymard M, Langlois M (1992): Occurrence and characterization of acyclovir-resistant herpes simplex virus isolates: Report on a two-year sensitivity screening survey. Journal of Medical Virology 36:1–12.

Orita M, Suzuki Y, Sekiya T, Hayashi K (1989): Rapid and sensitive detection of point mutations and DNA polymorphism using the polymerase chain reaction. Genomics 5:874–879.

Parker AC, Craig JIO, Collins P, Oliver N, Smith I (1987): Acyclovir-resistant herpes simplex virus infection due to altered DNA polymerase. Lancet ii:1461.

Reichman RC (1984): Herpes simplex virus infection. European Journal of Clinical Microbiology 3:399–405.

Richards DM, Carmine AA, Borgden RN, Heel RC, Speight TM, Avery GS (1983): Acyclovir: A review of its pharmacodynamic properties and therapeutic efficacy. Drugs 26:378–438.

Sacks SL, Wanklin RJ, Reece DE, Hicks KA, Tyler KL, Coen DM (1989): Progressive esophagitis from acyclovir-resistant herpes simplex: Clinical roles for DNA polymerase mutants and viral heterogeneity? Annals of Internal Medicine 111:893–899.

Sakaoka H, Kurita K, Iida Y, Takeda S, Umene K, Kim YT, Ren CS, Nahmias AJ (1994): Quantitative analysis of genomic polymorphism of herpes simplex virus type 1 strains from six countries: Studies of molecular evolution and molecular epidemiology of the virus. Journal of General Virology 75:513–527.

Shiota H, Naito T, Mimura Y (1987): Antiherpes simplex virus (HSV) effect of 9-(1,3-dihydroxy-2-propoxymethyl)guanine (DHPG) in rabbit cornea. Current Eye Research 6:241–245.

Shiota H, Kudo E, Mimura Y, Itakura M (1996): Mutation of the thymidine kinase gene in a ganciclovir-resistant strain of herpes simplex virus type 1 (HSV-1) detected by PCR-SSCP and PCR-direct sequencing. Biomedical Research 17:9–14.

Suzutani T, Koyano S, Takada M, Yoshida I, Azuma M (1995): Analysis of the relationship between cellular thymidine kinase activity and virulence of thymidine kinase-negative herpes simplex virus types 1 and 2. Microbiology and Immunology 39:787–794.

Wilhelmus KR (1987): Diagnosis and management of herpes simplex stromal keratitis. Cornea 6:286–291.